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Nicotine does not reduce *Nosema ceranae* infection in honey bees

H. P. Hendriksma^{1,2} · J. A. Bain¹ · N. Nguyen¹ · J. C. Nieh¹ Received: 26 January 2020 / Revised: 26 January 2020 / Accepted: 18 February 2020
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Abstract

Bee-collected pollen and nectar contain multiple phytochemicals that can have anti-pathogenic effects when ingested. For example, the plant alkaloid, nicotine, can reduce infections by the trypanosome gut parasite (*Crithidia bombi*) in bumblebees. Parasitized bumblebees may be drawn to nicotine and thereby self-medicate their infection. We tested the hypothesis that nicotine can reduce infections of a common microsporidian pathogen, *Nosema ceranae*, in the honey bee gut. We found, however, that that a field realistic exposure dose of 1 ppm nicotine was not preferentially consumed by *Apis mellifera* foragers fed live *Nosema* spores (5×10^4 spores per bee; $N=160$). One-day-old bees infected with *Nosema* (1×10^4 spores per bee; $N=160$) showed no repression of nosemosis over a chronically applied exposure gradient of 0, 10^{-2} , 10^{-1} , 10^0 , 10^1 , 10^2 , 10^3 or 10^4 ppm nicotine. Since imbibed nicotine may not effectively reach the spores in the bee gut, we conducted an in vitro experiment, in which *Nosema* spores were exposed up to 10^4 ppm nicotine in vials, rinsed of nicotine, and then fed to 1 day old bees (2×10^4 spores per bee; $N=216$). However, the in vitro nicotine-treated spores remained infectious. Nicotine did impair bee mortality at high concentrations. Dietary nicotine is evidently not a treatment for nosemosis, but future studies should continue to examine the role of phytochemicals and bee health.

Keywords *Apis mellifera* · Host–parasite interaction · Intracellular parasite · Nectar · Preference · Self-medication

Introduction

The Western honey bee, *Apis mellifera*, is one of the most abundant pollinator species in both natural and agricultural habitats, but, in many regions, honey bees have poor health (Garibaldi et al. 2013; Hung et al. 2018). In regions of Europe and the USA, honey bee colonies have shown declines due to a variety of stressors, including the spread of diseases (Goulson et al. 2015; Moritz and Erler 2016). A honey bee disease survey found that approximately 70% of managed colonies in the USA are annually infected with the microsporidian pathogen *Nosema*, at an average

of approximately 500,000 spores per infected bee (Rennich et al. 2012). Infection with *Nosema ceranae* is a common factor that likely contributes to observed colony losses in the USA (Cox-Foster et al. 2007).

There are two common microsporidian *Nosema* species that infect honey bees: *Nosema apis* (Zander 1909) and *Nosema ceranae* (Fries et al. 1996). *Nosema* infects the epithelial cells of the midgut of honey bee adults (Webster 1993) and larvae (Eiri et al. 2015). *Nosema ceranae* is native to Eastern honey bee colonies (*Apis cerana*) but has rapidly spread since the 1990s to *A. mellifera* colonies around the world and has evidently largely displaced *N. apis* (Klee et al. 2007; Papini et al. 2017; Sinpoo et al. 2018). Bees can be treated with the antibiotic fumagillin, which inhibits the enzyme methionine aminopeptidase-2 and thereby disrupts spore protein maturation (Sin et al. 1997; Huang et al. 2013). However, multiple countries prohibit fumagillin use because of the risk of residues in honey. In addition, the repeated use of this antibiotic may have contributed to parasite resistance, thus reducing options for treating *Nosema* infections (Tian et al. 2012; Huang et al. 2013). Identifying novel and effective treatments for Nosemosis is therefore critical.

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Although *N. ceranae* infection does not typically lead to colony death, it can weaken colonies and has synergistic effects with pesticide exposure and other causes of poor colony health (Pettis et al. 2012). *Nosema* can reduce vitellogenin (Vg) and increase Juvenile Hormone (JH) titers in bees—the reverse of what is normal for healthy individuals of nursing age (Goblirsch et al. 2013). Because the transition from nursing to foraging is regulated by the interaction of Vg and JH, young *Nosema*-infected bees begin to forage precociously, an atypical behavior (Paxton 2010). *Nosema* can thus contribute to the decline of brood care by nurses, the premature death of foragers, and poorer overall colony condition and health (Goblirsch et al. 2013).

Multiple plants produce secondary compounds that can be beneficial to animals. These secondary compounds in nectar are generally viewed as a byproduct of plant defensive strategies against herbivores (Stevenson et al. 2017), yet may also influence pollinators (Wright et al. 2013). Preferential collection of beneficial phytochemicals in pollen and nectar by pollinators could even be a self-medication behavior (Erler and Moritz 2016). For example, some insects can reduce parasite infections by consuming secondary compounds from plants. Woolly bear caterpillars infected with lethal endoparasitic larvae of tachinid flies ingested more parasitocidal alkaloids than unparasitized individuals (Singer et al. 2009). Fruit flies (*Drosophila melanogaster*) consumed alcohol to reduce infections by endoparasitoid wasps (Milan et al. 2012).

Honey bee colonies collect plant resins, creating propolis (Burdock 1998; Bankova et al. 2019) that they use to seal and strengthen the nest. Propolis also has antibacterial, antifungal and antiviral activity (Freitas et al. 2019) and is potentially produced in response to colony infections, to maintain colony health, or both (Simone-Finstrom and Spivak 2012; Gherman et al. 2014; Erler and Moritz 2016). Propolis may also help to suppress *Nosema* infection (Gherman et al. 2012; Yemor 2016), and other natural products may also help. Sunflower pollen collected by honey bee colonies reportedly reduced *Nosema* infections (Giacomini et al. 2018). Nicotine and other allelochemicals can suppress proliferation of the trypanosome gut parasite, *Crithidia bombi*, in bumblebees (Manson et al. 2010; Biller et al. 2015; Palmer-Young et al. 2017a; Richardson et al.

2015; Thorburn et al. 2015). Bernklau et al. (2019) recently demonstrated that caffeine, kaempferol, and *p*-coumaric acid (25 ppm) could reduce *N. ceranae* spore loads in honey bees, increasing the longevity of infected bees. Caffeine and nicotine are chemically similar alkaloids with relatively low molecular masses, having a carbon ring and one or more nitrogen atoms. Like caffeine, nicotine may be a suitable plant compound to use against *N. ceranae* infection in honey bees (Köhler et al. 2012; Baracchi et al. 2017).

Nicotine is commonly found in pollen and nectar of Solanaceae, a family of flowering plants with a worldwide distribution, which includes food and medicinal plants. Some members of this family are very attractive to honey bees because they provide easily accessible inflorescences and copious pollen and nectar: morning glory, jimsonweed, and sweet potato (Crane et al. 1984). Other members of this family depend upon buzz-pollination by bees: tomatoes, eggplant, bell and chili peppers (Buchmann 1983). Tobacco plants (*Nicotinia tabacum*) can be a source of high honey production for honey bee colonies, with 40 kg/colony/season reported in the USA (Espina Perez and Ordex Ros 1983). Bees can, therefore, be exposed to nicotine via pollen and nectar.

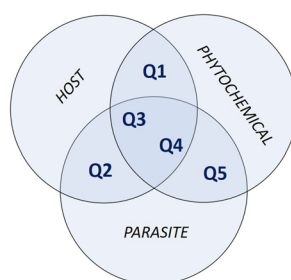
We hypothesized that nectar with nicotine is parasitocidal to *N. ceranae* in *A. mellifera*, and that infected bees may self-medicate by preferring nicotine laced sucrose solution to pure sucrose solution. We asked five research questions (Fig. 1). Q1: At what concentration does nicotine affect honey bee survival? Q2: Does *Nosema*-infection change sucrose consumption by honey bees? Q3: Do parasitized honey bees prefer food with nicotine more than food without nicotine? Q4: Can *N. ceranae* be suppressed within honey bees via nicotine consumption? Q5: Is nicotine parasitocidal to *N. ceranae* spores outside the host?

Materials and methods

Honey bees

We used honey bee colonies (*Apis mellifera ligustica*) kept at an apiary at the Biological Field Station at UC San Diego in La Jolla, California (32° 53' 13 N, 117° 13' 49 W). The

Fig. 1 Schematic overview of research questions. Potential interactions between a parasite (*Nosema*), its host (a honey bee), and a dietary phytochemical (nicotine) are listed in the Venn diagram with our five research questions shown to the right



Q1 (LD₅₀) Does the phytochemical affect host mortality?

Q2 (consumption) Does energy intake of hosts change by parasitism?

Q3 (choice) Does parasitism affect phytochemical intake by the host?

Q4 (in vivo) Is the phytochemical parasitocidal within the host?

Q5 (in vitro) Is the phytochemical parasitocidal outside the host?

colonies were managed following normal beekeeping practices. All colonies were queenright and healthy, as determined by standard inspection techniques. Different colonies were used for different experiments (see below).

Bees were incubated (Nor-Lake Scientific, model LRI-201WWW/0) under dark conditions in cages in the lab (see below). Test Q1 was performed at 24.7 ± 1.4 °C (mean \pm SD) with a Relative Humidity (RH) of $81.5 \pm 7.7\%$, as constantly monitored with data loggers (Onset HOBO, Wilmington, NC). Tests Q2 through Q5 were performed at 34.2 ± 0.2 °C with a RH of $65.1 \pm 5.4\%$.

Nosema spores

Nosema ceranae spores came from a stock of infected bees continuously renewed and maintained in bees kept in an incubator. Spores were collected by dissecting midguts from ten infected bees, and homogenizing the guts in 100 µl deionized (DI) water in a 1.5 ml microcentrifuge tube using a Kontes electric pestle. Homogenates were strained with a Buchner funnel and two filter papers with 2.5 µm pore size (Grade 5; Whatman) into a 100 ml Erlenmeyer flask under vacuum. The filtrate was transferred into 1.5 ml microcentrifuge tubes and centrifuged at 10^4 rpm (6702g) for 6 min (Eppendorf 5415D Centrifuge). After discarding supernatants, the spore pellets were combined into one spore concentrate with a volume of 0.5 ml. Spore concentrations were measured with a Neubauer Improved Hemocytometer on a Zeiss Microscope under 400× magnification (Cantwell 1970). Dissection, extraction, and concentration of fresh spores occurred on the same day as the choice trial (§2.4) and the in vitro trials (§2.6). However, for the in vivo trial (§2.5) we used a stored spore stock that had been refrigerated for 3 weeks at 4 °C. To determine spore identity, we used standard DNA extraction methods, PCR amplification with the primer pair NoscRNAPol-F2 and NoscRNAPol-R2

(Gisder and Genersch 2013), and amplicon sequencing and confirmed via GenBank sequence comparison that the spores were *N. ceranae*.

Q1: Nicotine and dimethoate toxicity to honey bees

We analyzed bee survival to calculate nicotine and dimethoate LD₅₀ values at 24 h and 48 h after feeding. Dimethoate is an organophosphate insecticide and is a standard reference toxin used to validate bioassays (Medrzycki et al. 2013). The LD₅₀ is the amount of ingested substance that kill 50% of a test population within a specified time period. This toxicity value can be based on acute (one-time) exposure or chronic (constant) exposure and is commonly expressed in µg/bee for honey bees, which is an alternative to the expression in mg/kg body weight (Johnson 2015). Our toxicity tests followed the guidelines of the European and Mediterranean Plant Protection Organization (EPPO) and the International Commission for Plant–Pollinator Relationships (ICPPR). Specifically, we used the OECD guidelines for the acute oral toxicity tests on honey bees (OECD 1998; Medrzycki et al. 2013).

We designed nicotine test concentrations based upon Detzel and Wink (1993) who report an oral 48 h LD₅₀ of 0.2% (2×10^3 ppm nicotine), for honey bees chronically fed nicotine. Test solutions were prepared using DI water with 1.5 M analytical-grade sucrose (Fisher Scientific, Fair Lawn, NJ). Liquid (–)-Nicotine was obtained from Sigma-Aldrich (N3875-5ML; Milwaukee, WI, USA). For dimethoate toxicity testing we used a concentration range based upon an acute oral 24 h LD₅₀ for honey bees of 0.10–0.35 µg dimethoate (OECD 1998). Dimethoate was obtained from Fluka Chemie (Switzerland). All test levels were in a geometric concentration series with spacing factor of two (Table 1).

Brood combs without worker bees were incubated for 20 h in a nuc box at 33 °C and 70% RH. The emerged bees

Table 1 LD₅₀ experimental design

Formulation	Concentration	Dose/bee
10 µl nicotine + 7990 µl sucrose soln.	0.25 µl/200 µl (1250 ppm)	12.6 µg
10 µl nicotine + 3990 µl sucrose soln.	0.50 µl/200 µl (2500 ppm)	25.3 µg
25 µl nicotine + 4975 µl sucrose soln.	1.00 µl/200 µl (5000 ppm)	50.5 µg
25 µl nicotine + 2475 µl sucrose soln.	2.00 µl/200 µl (10,000 ppm)	101.0 µg
50 µl nicotine + 2450 µl sucrose soln.	4.00 µl/200 µl (20,000 ppm)	202.0 µg
16.14 mg dimethoate + 10 ml sucrose soln.; 1:800	0.4035 µg/200 µl (2 ppm)	0.0202 µg
16.14 mg dimethoate + 10 ml sucrose soln.; 1:400	0.8070 µg/200 µl (4 ppm)	0.0404 µg
16.14 mg dimethoate + 10 ml sucrose soln.; 1:200	1.6140 µg/200 µl (8 ppm)	0.0807 µg
16.14 mg dimethoate + 10 ml sucrose soln.; 1:100	3.2280 µg/200 µl (16 ppm)	0.1614 µg
16.14 mg dimethoate + 10 ml sucrose soln.; 1:50	6.4560 µg/200 µl (32 ppm)	0.3228 µg
50 g sucrose + 50 g DI water	50% w/v sucrose	0 (control)

The acute oral toxicity of nicotine and dimethoate on honey bees was tested with the following doses. The density of 1.01 g/cm³ nicotine was used to convert 10 µl exposure volumes per bee to mg doses

were collected in four plastic cages (500 cm³) and offered solutions of 50% w/v sucrose solution, ad libitum. All bees were 14 days old when they were tested, the average age at which honey bees begin foraging (Schippers et al. 2006). 2 h before the test, bees were transferred into 36 disposable 80 cm³ paper test cages, with a ventilated bottom and a clear plastic sheet at the front. Eleven treatment doses (Table 1) were replicated with four colonies and 20 bees per cage (11 × 4 × 20 = 880 bees). Each cage contained 200 µl of the solution in the caps of two 1.5 ml microfuge tubes, thus providing 10 µl solution per bee, on average. The bees consumed all test solutions within 4 h. The emptied caps were then removed and two 5 ml syringes with 50% (w/v) pure sucrose solution were introduced. Each syringe had the tip cut off to create a 2 mm diameter opening for feeding. Sucrose solution was provided ad libitum. Mortality was recorded daily over 4 days.

Q2 and Q3: *Nosema* infection, nicotine choice and sucrose consumption by bees

Forager bees were collected upon their return to their respective hives for preferential nicotine consumption tests. We targeted foragers because they can choose what to bring back to the hive, unlike young hive-bees. The foragers were kept in 16 paper cages (160 bees of four colonies; ten bees per cage). All bees were experimentally infected with *Nosema* spores by feeding 5 × 10⁵ spores per cage (5 × 10⁴ per bee). All cages were given two feeding syringes with 50% (w/v) sucrose solution; one with nicotine at 1 ppm, and the other without. Solution consumption was measured by weighing all syringes every second day. The positions of the control syringe and nicotine treatment syringe were swapped after weighing, to counter potential spatial bias effects within cages. Syringes and solutions were all replaced at day 7. Evaporation under test conditions was measured with refractometry: the solutions were 43.6°Bx at day 0 (50% w/v), yet 51.0°Bx at day 5, indicating a daily evaporation loss of mean 1.8 ± 0.4% (N = 20 syringes). We did not apply a data correction for evaporation since solutions with and without nicotine did not differ in evaporation loss (2-sided *t* test: *t* = 0.53, *p* = 0.40). Bee survival was recorded daily, and dead bees were removed. Per cage (N = 16), one bee was randomly collected at day 14 to verify successful *Nosema* infection (Cantwell 1970).

Q4: *Nosema* virulence over a nicotine gradient fed to bees in vivo

We tested if nicotine could reduce *Nosema* infection when fed to bees ($N_{\text{bees}} = 160$, $N_{\text{paper cages}} = 8$, $N_{\text{colony}} = 1$). In this in vivo experiment, we fed all bees with spores. After *Nosema* spores were fed to newly emerged bees (2 × 10⁵

spores per cage; mean 10⁴ spores per bee), the consumption and mortality of the bees were recorded over 14 days. Bees were fed nicotine solutions chronically with a different concentration for every cage. A stock solution of 10⁴ ppm (1 mg/l) nicotine was made in a 50% sucrose solution (w/v) prepared with DI water. Subsequently we made 1:10 serial dilutions (10⁴, 10³, 10², 10¹, 10⁰, 10⁻¹, 10⁻² ppm). After 14 days of chronic exposure, *Nosema* infection in bees was assessed for N = 72 midgut samples, with spore counts performed in duplicate and the average calculated per bee.

Q5: *Nosema* exposure to high nicotine concentrations, in vitro

Freshly collected *Nosema* spores were exposed to nicotine concentrations in vitro, then washed, and subsequently fed to bees to determine their ability to infect bees. For example, a 2 × 10⁴ ppm nicotine solution was made with DI water, then mixed with an equal volume of fresh *Nosema* spore extract, thus creating a 10⁴ ppm nicotine/spore mix. The mixtures were incubated at room-temperature (21 °C) for 60 min at 300 rpm (Eppendorf Thermomixer 5350). After incubation, spores were spun down into a pellet by centrifugation for 10 min at 12 × 10³ rpm (16,128g, a higher speed than our initial purification method to ensure that we pelleted as many of the treated spores as possible). Supernatants were carefully removed and pellets were resuspended in 1 ml DI water to rinse the spores. Spores were then re-pelleted in the centrifuge following the prior steps to discard the supernatant water. The rinsed spores were resuspended in 50% w/v sucrose solution so that 7 µl of solution contained approximately 2 × 10⁴ spores (Table S1).

Test bees were collected within 24 h of emergence from brood combs (N = 6 colonies) and incubated overnight at 34 °C. Each bee was individually fed the pre-treated spores by inserting a pipette tip with 7 µl solution through the lid of a 13 ml clear styrene snap cap vial (23 mm diameter × 44 mm high). The vials were placed on trays in a dark incubator at 34 °C. Approximately 50% of bees had consumed their dose in 1 h, and 95% of bees had imbibed the dose within 4 h. We discarded bees that had not fully consumed their full dose (fluid still visible in the pipette tip). After exposure, ten bees were grouped per treatment and colony in 500 cm³ plastic cages. Each cage, with ventilation and feeding holes, was equipped with two feeding syringes with 50% (w/v) sucrose solution. After 14 days, all bees were frozen at -20 °C to assess infection results via hemocytometer spore counting (Cantwell 1970).

Each spore pre-treatment batch was replicated six times (Table 2). Every spore feeding concentration was checked (N = 18; Table S1). A fumagillin positive control treatment at 25 ppm followed the recommended concentration for colony treatment (Huang et al. 2013; Palmer-Young et al.

Table 2 *Nosema* spore pre-treatment with nicotine

Honey bee infection with <i>Nosema</i>
No <i>Nosema</i> spores (control 1)
Spores pre-exposed to Fumagillin (control 2)
Spores pre-exposed to DI water (0 ppm)
Spores pre-exposed to 1 ppm nicotine
Spores pre-exposed to 10 ² ppm nicotine
Spores pre-exposed to 10 ⁴ ppm nicotine

To assess if nicotine is parasiticidal in vitro, *Nosema* virulence in bees was tested after a spore pre-treatment with nicotine

2017b). In one of our six colonies, we found *Nosema* infection in the negative control bees and therefore this colony was removed from the analysis (Table S1).

Statistical analyses

Data analyses were performed with JMP Pro 13.1.0 software. Probit analyses were used to calculate 24 h and 48 h LD₅₀ values. Measures of uncertainty were estimated with SE, and measure differences were indicated with 95% confidence intervals (Finney 1952). A Schneider-Orelli (1947) adjustment for background mortality was applied. We report our results as mean ± standard error.

Forager consumption and choice among feeding solutions, with or without 1 ppm nicotine, after experimental *Nosema* infection, were analyzed with Linear Mixed Effects Models. Daily solution consumption was analyzed as the response variable, with nicotine (two levels; 0 and 1 ppm) and colony background (four levels) as fixed factors, and time as a covariate (11 time points). Following standard procedures, we first ran models with nicotine × time and nicotine × colony interactions but removed the interactions if they were not significant. Pairwise post hoc comparisons

were made with Tukey's Honestly Significant Difference (HSD) tests (Fig. 3).

For the in vivo experiment, sucrose consumption and 48 h bee survival per cage ($N=8$) were correlated with linear regression (Fig. 4a, b). Midgut spore counts between nicotine levels were compared with a non-parametric test (Kruskal–Wallis). We made pairwise comparisons with Mann–Whitney tests and Dunn's Multiple Comparison tests. We performed a linear regression over the five nicotine concentration levels (0 ppm excluded) versus the midgut *Nosema* spore counts (Fig. 4c).

The effect of pre-treating *Nosema* spores in vitro, before honey bee infection with nicotine, fumagilin or DI water, was analyzed with Generalized Mixed Effects models. The response variable, midgut spores 14 days post-infection, were log₁₀ transformed (count + 1) and analyzed with treatment as an explanatory variable (six levels), and colony (five levels) nested in replicate trials (two levels) as a random factor. Due to our rinsing protocol, bees were fed slightly different amounts of spores in this experiment. We, therefore, tested for an effect of the number of spores fed to bees but removed this variable because it was not significant (see Table S1). Pairwise post hoc comparisons were made with Tukey's HSD tests (Fig. 5).

Results

Nicotine toxicity to honey bees (Q1) The acute oral 48 h LD₅₀ dose was 80.5 ± 22.7 µg nicotine per bee, and the organophosphate insecticide dimethoate was 2000 times more toxic at a dose of 0.041 ± 0.014 µg/bee (Fig. 2, Table 3). The background mortality was 0% at 24 h, 5% at 48 h, 8.8% at 96 h, for $N=80$ unexposed bees. The confidence intervals indicated that the 24-h and 48-h toxicity values did not significantly differ (Table 3).

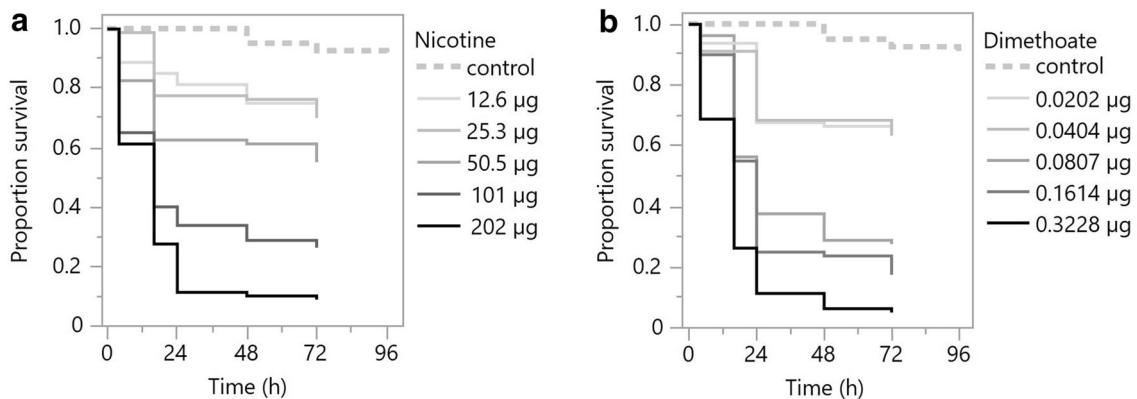


Fig. 2 Survival of bees after exposure to different concentrations of nicotine (a), and different concentrations of dimethoate as a positive toxicity control (b). These survival data were used to calculate the final 24 h and 48 h LD₅₀ values (Table 3)

Table 3 Acute oral toxicity of nicotine and dimethoate to honey bees

LD ₅₀	Nicotine (Fig. 2a; <i>N</i> =480 bees)	Dimethoate (Fig. 2b; <i>N</i> =480 bees)
24 h	65.2 µg, 95% CI [46.0–92.3], <i>N</i> =24 cages Mean 78.7 µg ± 23.6 SE, <i>N</i> =4 colonies	0.053 µg, 95% CI [0.037–0.077], <i>N</i> =24 cages Mean 0.053 µg ± 0.019 SE, <i>N</i> =4 colonies
48 h	78.6 µg, 95% CI [53.5–115.4], <i>N</i> =24 cages Mean 80.5 µg ± 22.7 SE, <i>N</i> =4 colonies	0.098 µg, 95% CI [0.072–0.135], <i>N</i> =24 cages Mean 0.041 µg ± 0.014 SE, <i>N</i> =4 colonies

Table 4 Overview of honey bee experiments with experimental *Nosema* infection

<i>Nosema</i> trials	Nicotine exposure (ppm)	Bee age	Spores fed	Mean spores (million)	Amplification
Choice, §2.4	0 and 1	Forager	50 × 10 ³ /bee	7.15 ± 2.47 SE, <i>N</i> =16	×142
In vivo, §2.5	0, 10 ⁻² , 10 ⁻¹ , 10 ⁰ , 10 ¹ , 10 ² , 10 ³ , 10 ⁴	0–14 days	10 × 10 ³ /bee	1.93 ± 0.21 SE, <i>N</i> =72	×204
In vitro R1, §2.6	0, 1, 10 ² , 10 ⁴	0–14 days	20 × 10 ³ /bee	6.28 ± 0.97 SE, <i>N</i> =36	×315
In vitro R2, §2.6	0, 1, 10 ² , 10 ⁴	0–14 days	14 × 10 ³ /bee	1.52 ± 0.20 SE, <i>N</i> =36	×107

Fig. 3 Consumption and choice of infected honey bee foragers feeding on sucrose with and without nicotine. Shown are (a) the mean sucrose solution consumption in mg per bee per day with SE error bars for *N*=4 colonies, and (b) means with SE error bars over *N*=11 time-points. Different letters indicate significant differences (Tukey's HSD Test, *p* < 0.05)

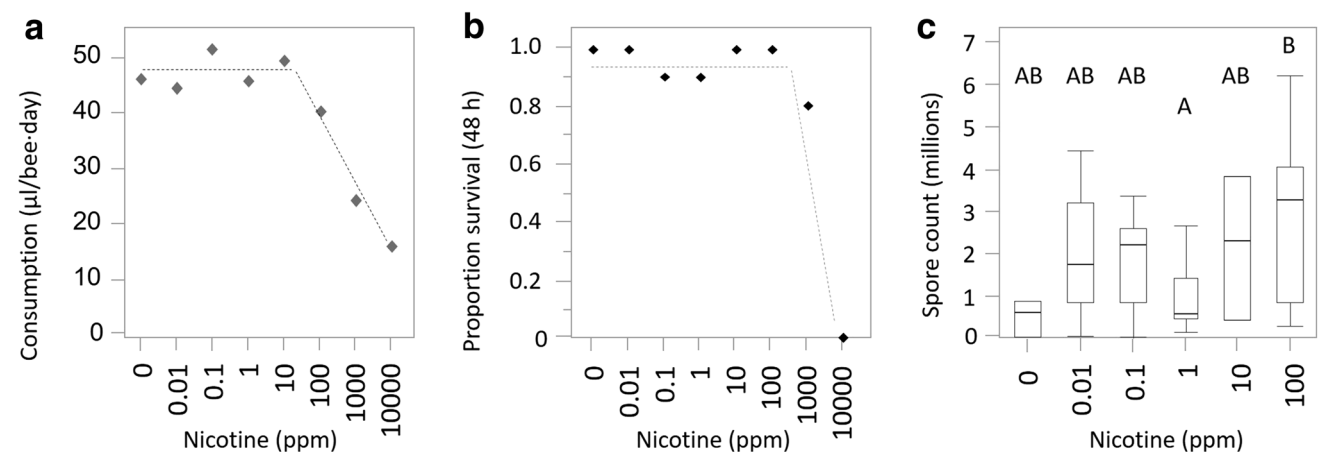
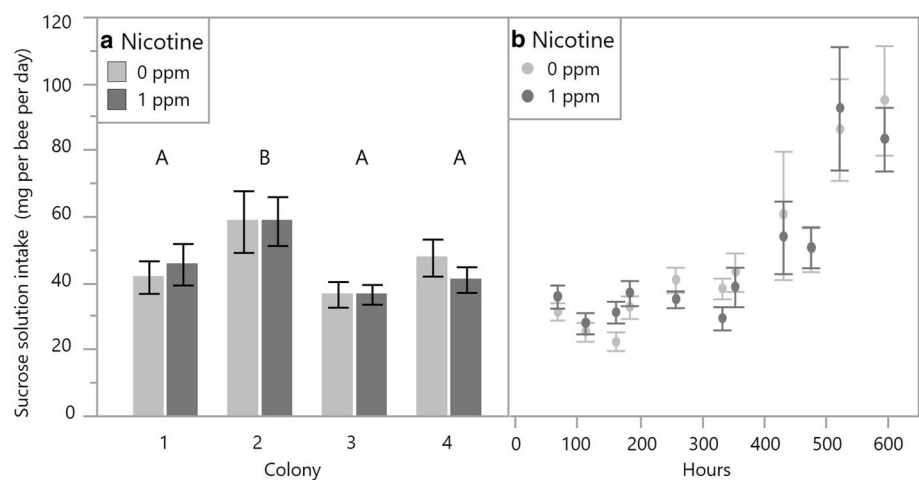


Fig. 4 Honey bee infection with *Nosema*, with in vivo exposure to nicotine. *Nosema* parasitized bees, in eight cages, were chronically exposed to nicotine over a 10⁷ fold increasing concentration range for 14 days. **a** Bees exposed to nicotine doses higher than 10 ppm reduced their consumption (grey dashed line). **b** Survival decreased sharply for nicotine concentrations > 100 ppm (dashed black line). **c**

Median and quartile spore counts are shown by boxplots, with error whiskers indicating minimum and maximum values for *N*=72 mid-gut spore counts. Different letters indicate significant differences between treatments (Dunn's Multiple Comparison Test, *p* < 0.05). No spore counts for nicotine doses 1000 and 10,000 ppm due to bee mortality

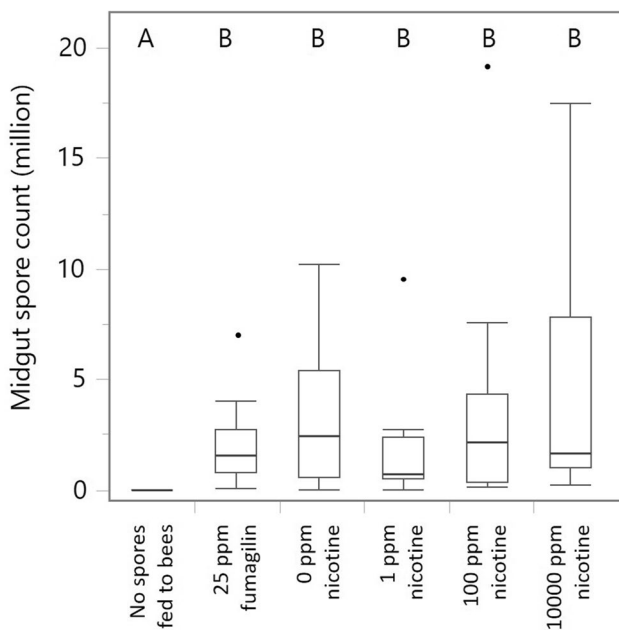


Fig. 5 *Nosema* spore pre-treatment, in vitro. The boxplots show medians and quartiles of *Nosema* spore counts, 14 days post infection, of 108 honey bee midguts (Table S1). Error whiskers indicate minimum and maximum values with outlier data points as dots. Different letters indicate a significant difference (Tukey HSD test, $p < 0.05$)

***Nosema* and nicotine consumption by honey bees (Q3)** Our *Nosema* spores did infect bees. Midgut spore counts showed that bees fed spores had 107- to 315-fold more spores by the end of the experiment than they were fed (Table 4). Bees infected with *Nosema* did not significantly consume more 1 ppm nicotine sucrose solution than the 0 ppm nicotine sucrose solution (Fig. 3; $F_{1,328} = 0.05$, $p = 0.82$). Consumption did vary based on colony background: colony 2 consumed significantly more than other colonies (Fig. 3a; $F_{3,328} = 8.27$, $p < 0.001$). Consumption increased significantly over time (Fig. 3b; $F_{1,328} = 102.1$, $p < 0.001$). The interactions for nicotine \times colony ($F_{3,330} = 0.36$, $p = 0.78$) and nicotine \times time ($F_{3,329} = 0.81$, $p = 0.37$) were not significant.

***Nosema* and nicotine doses in vivo (Q4)** The young bees infected with *Nosema* and fed 10^4 ppm nicotine (in vivo experiment), consumed a mean of 16.2 μ l sucrose solution over 2 days (Fig. 4a), with 0% bee survival over a 48 h period (Fig. 4b). At a far lower nicotine dose (10^2 ppm), bees consumed an average of 24.4 μ l per day (Fig. 4a), and 80% of the bees survived over 48 h (Fig. 4b)—although all of these bees were dead within 5 days. Bee consumption and survival were correlated ($R^2 = 0.68$, $F_{1,6} = 12.5$, $p = 0.012$). Midgut *Nosema* spore counts 14 days post infection are shown with boxplots (Fig. 4c). The spore counts differed according to nicotine concentrations ($\chi^2(5) = 13.1$, $p = 0.022$), with a significant difference between 1 and 100 ppm nicotine ($Z = 3.0$,

$p = 0.04$), however, the *Nosema* spore count did not significantly increase or decrease over the five nicotine dose levels (regression; $p > 0.05$).

Nicotine and Nosema in vitro (Q5) Honey bees can metabolize ingested nicotine (Du Rand et al. 2017a, b), thus nicotine may not reach the sites of *Nosema* infection. We, therefore, also tested the effect of pre-treating *Nosema* with nicotine in vitro (outside of the honey bee host), a method that also enabled us to test supra-lethal concentrations. Over 97% of bees fed the pre-treated *Nosema* spores were successfully infected (2.94 million midgut spores on average), which was significantly different from the negative control group in which 0% of bees were infected ($F_{5,80} = 87.3$, $p < 0.001$; Tukey HSD test, $p < 0.05$). Interestingly, pre-treated spores that had been exposed to water (0 ppm nicotine), or fumagillin, were just as infective as spores that had been exposed to 1, 10^2 or 10^4 ppm nicotine (Fig. 5).

Discussion

Multiple animals can use natural compounds such as alkaloids to counter pathogen infections. We tested the hypothesis that the alkaloid, nicotine, can help reduce infections of the common gut parasite *N. ceranae* in the honey bee, *A. mellifera*. However, *Nosema* infection levels were not affected when we fed nicotine to bees directly, or first pre-treated spores with nicotine and then fed these treated spores to bees—despite testing a wide range of nicotine concentrations that ranged from field realistic (1 ppm) to fatal (10^4 ppm; 100% bee mortality).

With an acute oral 48 h- LD_{50} of 53.5–115.4 μ g (95% CI) per honey bee, nicotine is classified as essentially non-toxic to bees (Environmental Protection Agency guidelines, $LD_{50} > 11$ μ g/bee). Following 48 h of chronic exposure (Fig. 4b), our median bee survival at 10^3 and 10^4 ppm nicotine in sucrose solution was similar to the LD_{50} at 2000 ppm reported by Detzel and Wink (1993). In comparison, dimethoate was 2000 times more toxic than nicotine (Table 4). Dimethoate is classified as highly toxic to bees (i.e., $LD_{50} < 2$ μ g active ingredient per honey bee). With respect to our first research question (Q1), nicotine, at natural levels found in nectar and pollen, is highly unlikely to harm bees.

Nosema contributes to poor honey bee health and global losses of honey bee colonies (Burnham 2019) and can reduce brood and honey production (Botías et al. 2013). *Nosema* infection may change bee behaviors, such as task allocation (Goblirsch et al. 2013; Lecocq et al. 2016), increase bee activity (Alaux et al. 2014; Wells et al. 2016), and cause infected bees to spend more time outside of their colony but engage in shorter foraging bouts (Kralj and Fuchs 2010; Wolf et al. 2014; Dosselli et al. 2016). Regarding the

research question Q2, we found that bees steadily increased their sucrose consumption after experimental infection (Fig. 3b). Likewise, Naug and Gibbs (2009) found increased hunger in *Nosema* infected workers, and Mayack and Naug (2010) found a decline in hemolymph sugar levels in *Nosema* infected foragers. *Nosema* lacks mitochondria and is, therefore, heavily dependent on the ATP production of its host (Gómez-Moracho et al. 2017; Mayack and Naug 2009). The observed increased feeding may, therefore, be caused by decreased energy levels due to infection stress.

At low doses, nicotine may potentially enhance reward association in bees (Thany and Gauthier 2005; Gauthier 2010; Stevenson et al. 2017), and foraging bumblebees are evidently attracted to low nicotine levels (Manson et al. 2010; Thorburn et al. 2015; Baracchi et al. 2017). At the field-realistic dose of 1.6 ppm, bumblebees were able to detect nicotine (Tiedeken et al. 2014). Our honey bees infected with *Nosema* showed no preference for feeding on 1 ppm versus 0 ppm nicotine in sucrose solution (Fig. 3a). However, the field realistic dose of 1 ppm is relatively low compared to nicotine concentrations that elicited honey bee attraction or repulsion (Köhler et al. 2012; Singaravelan et al. 2005, 2006). We note that nicotine can occur naturally at concentrations higher than 1 ppm, i.e., 5 ppm in nectar and 23 ppm in pollen (Tadmor-Melamed et al. 2004; Singaravelan et al. 2005, 2006). Furthermore, the dose-dependent deterrence of nicotine is stronger at a lower nectar concentration, such as 0.65 M sucrose (Köhler et al. 2012), but we tested nicotine with a relative high concentration of 1.5 M sucrose solution that may have increased bee attraction and decreased a repelling effect of nicotine. However, our results did not show that honey bees preferred nicotine solutions over controls (Fig. 3a), and infected bees did not increase their uptake of sucrose solutions when nicotine concentrations were higher (Fig. 4a). This strongly suggests that *Nosema* infection did not affect nicotine intake by honey bees (Q3).

Given that nicotine did also not actively suppress *Nosema* infection (Figs. 4c, 5), and merely increased mortality (Figs. 2a, 4b), it is not surprising that infected bees did not prefer nicotine. Under no-choice feeding conditions (Fig. 4), higher concentrations of nicotine (10^2 , 10^3 and 10^4 ppm) were aversive to bees. Similarly, bumblebees were deterred by 50 ppm nicotine in sucrose solution (Baracchi et al. 2017). Bee avoidance or rejection behavior may be explained by their gustatory ability to detect nicotine (De Brito-Sanchez 2011), by higher-level effects based upon acetylcholine receptor-based pathways (Gauthier 2010), or both.

Fumagillin pre-treatment of *Nosema* spores in vitro did not impair the ability of spores to infect bees (Fig. 5). This is perhaps not surprisingly given that the antibiotic fumagillin acts intracellularly by disrupting *Nosema* merogony. Fumagillin, therefore, affects *Nosema* in its vegetative stage,

after a spore infects a bee midgut cell (Sin et al. 1997; Huang et al. 2013) to kill *Nosema* spores directly (Badowska-Czubik et al. 1984). Our pre-exposure of *Nosema* spores to nicotine at 10^0 , 10^2 and 10^4 ppm, before feeding them to bees, had no effect on *Nosema* virulence (Q5, Fig. 5). In contrast, the gut parasite *Crithidia bombi*, in a similar in vitro exposure experiment with nicotine, did slightly delay the development of this parasite after infecting its bumblebee host (Baracchi et al. 2015). One difference may be that *C. bombi* reproduces extracellularly in the gut lumen, whereas *Nosema* develops intracellularly, e.g., within epithelial cells, where concentrations of imbibed nicotine are likely lower than in the lumen. In addition, *Nosema* is a prokaryote and *Crithidia* an eukaryote. Further, *C. bombi* lacks a rigid spore wall which protects *Nosema* spores from abiotic stressors such as heat and desiccation (Fenoy et al. 2009), and potentially from compounds such as nicotine.

The methods used to feed bees *Nosema* spores can have variable effects on the final infection levels and on bee survival (Milbrath et al. 2013; Urbietta-Magro et al. 2019). Although we infected newly emerged bees and foragers (Table 4) and used both group-level spore feeding (§2.4, §2.5) and individual bee feeding (§2.6), the final spore loads were comparable between our experiments and were similar to those found in other studies (Paxton et al. 2007; Maistrello et al. 2008; Rubanov et al. 2019). We note that fresh *N. ceranae* spores are thought to be relatively fragile and susceptible to temperature stress (Fries 2010), hence the preference for using fresh spore extracts for infection trials. However, for the in vivo experiment, a three-week old extract of *N. ceranae* maintained at 4 °C resulted in a 204-fold spore amplification rate (Table 4). Perhaps surprisingly, given hypothesized spore fragility, in vitro pre-exposure to high nicotine solutions, and a subsequent water washing of spores, did not affect *Nosema* virulence (Table 4; 107-fold and 315-fold amplification), as compared to unmanipulated fresh spore extracts used for the choice and in vivo experiments (Table 4; 142-fold and 204-fold, resp.). These results show that *Nosema* spores can be robust and remain virulent despite intense handling—as befits a widespread and common pathogen.

Multiple studies have suggested that nicotine could help honey bees or bumblebees fight off gut parasites (Köhler et al. 2012; Baracchi et al. 2015; Biller et al. 2015; Richardson et al. 2015; Thorburn et al. 2015; Palmer-Young et al. 2017a; De Roode and Hunter 2018). Our results show that dietary nicotine does not suppress *N. ceranae* infections in honey bees and bees did not exhibit self-medication. Yet honey bees, like many other insects, can be harmed by nicotine toxicity, via nicotinic acetylcholine receptor (nAChR) activation, which promotes action potentials in postsynaptic nerve cells (Johnson 2015). Notwithstanding, other phytochemicals may suppress nosemosis and should be tested

given the importance of finding new anti-microsporidian agents (Holt and Grozinger 2016; Burnham 2019).

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